SYNTHESIS OF A NOVEL PARAMAGNETIC AMINO ACID DERIVATIVE (EPM-5) FOR LABELING DIFFERENT MACROMOLECULES AND SYSTEMS

FIELD OF THE INVENTION

This invention relates to the chemical synthesis of a new paramagnetic β-amino

5   acid derivative containing a stable nitroxide radical moiety inserted in its pyrrolidine

structure and in which the 9-fluorenylmethyloxycarbonyl group (Fmoc) was chosen as its

amine function protecting group. This paramagnetic compound is a new type of spin probe

(or spin label) and can be used as alternative report molecule for labeling peptide

sequences, other macromolecules and systems where electron paramagnetic resonance

10   spectroscopy (EPR) can be applied. This compound can be used also for other

spectroscopic methods such as fluorescence and nuclear magnetic resonance since its

paramagnetism may affect the spectra of these methodologies. Due to the presence of both

carboxyl and amine groups in its structure, this organic compound may be used for labeling

a great variety of other molecules or systems containing reactive functions for these two

15   groups.

BACKGROUND AND SUMMARY OF THE INVENTION

The intermediate for the synthesis of the amino acid derivative of this invention

contains the structure 2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-amine-4-carboxylic acid,

henceforth denominated as Poac, synthesized more than two decades ago (see, Tetrahedron

20   491-499 [1965] and Bull. France, 3, 815-817 [1967]). The Poac derivative containing the

Fmoc protecting group (2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-(9-

fluorenylmethyloxycarbonyl)-amine-4-carboxylic acid) is the novel spin probe derivative of

the present invention, which also includes the synthesis and use in EPR of this spon probe

derivative. This new compound allows the insertion of Poac as a usual amino acid at any

25   position of a peptide sequence, and its denomination will be Fmoc-Poac or EPM-5 in this

application. The chemical structure of this paramagnetic molecule is represented in Figure

1.

Electron paramagnetic resonance (EPR) [described in *Biological Magnetic Resonance*, Berliner, L. J. and Reuben, J., eds., Plenum Publishing, New York, (1989)], is a modern and very useful spectroscopic method because it allows the study of paramagnetically labeled macromolecules or biological systems regarding their

5   conformation, mobilities, inter- or intra-molecular interactions, structuring states, and the like. The wide spectrum of EPR application is already detailed in the literature (see, *Free Nitroxyl Radical*, Rozantsev, E.G., Ulrich, H., ed., Plenun Press, London, 1970), where a great variety of spin labels, i.e., chemical compounds which are paramagnetic due to the presence of an unpaired electron in their structure, is listed. They are, therefore, a class of

10   free radicals but are necessarily stable under conditions around normal temperature and physiological pH and also allow several chemical reactions or experiments without affecting their free radical moiety.

Amongst the most commonly used spin labels one can pick out the nitroxide group-containing molecules and where the unpaired electron locates. The most significant

15   progress in the EPR field for labeling of relevant biological structures such as peptides and proteins was achieved with this class of spin probes. Almost two decades ago appeared the first EPR application in the solid phase peptide synthesis methodology [(*The Peptides: Analysis, Synthesis and Biology*, vol. 2, Academic Press, New York, (1980)]. This approach was introduced by our group using, instead, another nitroxide-containing spin

20   label, denominated at that time as Toac (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amine-4-carboxylic acid), which protected its amine function with the acid labile tert-butyloxycarbonyl (Boc) group [(see, Braz. J. Med. Biol. Res. *14*, 173, (1981) and Biochim. Biophys. Acta, *742*, 63, (1983)]. Thus the Boc-Toac spin probe was the first in the literature used to label a peptide sequence as an amino acid. However, due to chemical

25   particularities of the peptide synthesis methodology, it was only possible to couple the Toac group at the peptide N-terminal position. To overcome this shortcoming, an alternative

strategy was published by us which finally allowed the insertion of the spin label internally to the peptide sequence [see, J. Am Chem. Soc. *115*, 11042 (1993)].

An impressive increase in the application of EPR for peptide chemistry field was further observed in reports investigating peptide conformational properties [v.g. J. Am.

5   Chem. Soc. 117, 10555 (1995); FEBS Lett. *375*, 239 (1995); Biopolymers *42*, 821 (1997)] or of peptidyl-resin solvation (Tetrahedron Lett. *375*, 239 [1995]; Biopolymers *42*, 821 [1997]). As an amino acid, Toac was introduced in different positions of some biologically active peptides such as angiotensin II and bradykinin, but a partial or total loss of their biological potencies were observed due to the introduction of a non natural compound in

10   their structures. [*Peptides 1996*, R. Ramage and R. Epton, eds., Mayflower Scientific Co. p. 673 (1998)].

However, we recently described the synthesis of a peptide hormone labeled with Toac where its biological potency was entirely preserved. [e.g., FEBS Lett. *446*, 45 (1999)]. This result was obtained with the tridecapeptide α-melanocyte stimulating hormone

15   analogue, owing to its potentialities in a great number of chemical-biological assays (this analogue is paramagnetic, naturally fluorescent and fully active, and was disclosed in commonly owned U.S. patent application Serial No. 09/935, 760, entitled "Paramagnetic And Active Analogue (EPM-2) Of Melanocyte Stimulating Hormone Containing Amino Acid-Type Stable Free Radical").

20   In spite of these results, one still remaining shortcoming in the use of Toac in peptide chemistry is the severe difficulty in coupling the subsequent amino acid residue of the peptide sequence during the synthesis. This difficulty seems to be due to the low nucleophilicity of the Toac amine group, whose pKa of 8 (when in free state) decreases to about 5.5 when bound to the N-terminal portion of a peptide chain [v.g Braz J. Med. Biol.

25   Res. *14*, 173 (1981) and Biochim. Biophys. Acta. *742*, 63 (1983)]. Several recouplings and an increase in the temperature of the coupling reaction are usually necessary to assure

complete incorporation of the subsequent amino acid of the desired peptide sequence [J. Am. Chem. Soc. *115*, 11042 (1993)].

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a depiction of the structure of Fmoc-Poac or EPM-5 according to this invention.

Figure 2 is a mass spectrum of Poac.

Figure 3 is a high performance liquid chromatography (HPLC) profile of Poac showing a single peak.

Figures 4A and 4B depict EPR spectra of Poac in water and Fmoc-Poac in dimethylformamide, respectively.

Figure 5 is a mass spectrum of Fmoc-Poac.

Figure 6 is a HPLC profile of Poac[7] showing a single peak.

Figure 7 is a mass spectrum of Poac[7]-angiotensin II.

Figures 8A, 8B and 8C depict EPR spectra of Poac[7]-angiotensin II at pH 3.0, 6.0 and 9.0, respectively.

DETAILED DESCRIPTION OF THE INVENTION

In order to overcome the limitations of the Toac probe to find an alternative spin label which may induce differentiated conformational constraints in peptide structures in accordance with this invention, we synthesized Fmoc-Poac according to the synthetic route shown below that is partially described in Tetrahedron, 491-499 (1965); Bull. Soc..Chim. France, *3*, 815 (1967):

| P1 | P2 | P3 | P4 |
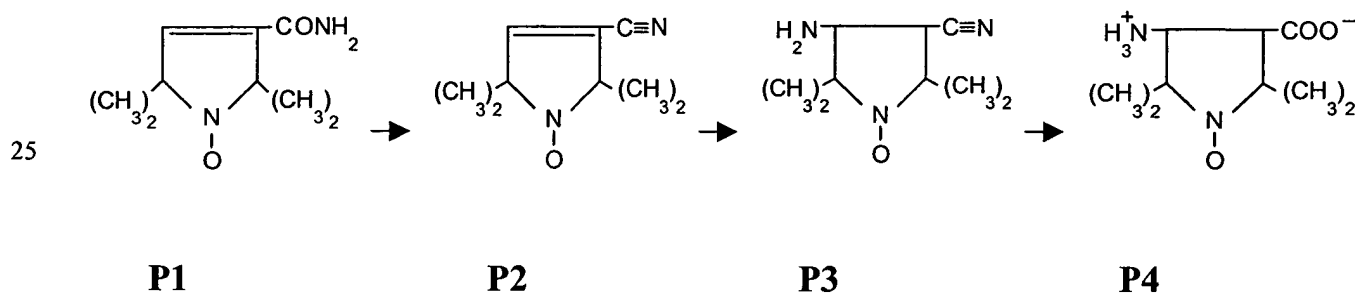
Step 1 - Synthesis of 2,2,5,5-tetramethylpyrroline-1-oxyl-3-cyano (P2)

This product was synthesized by treating compound P1 (from Sigma Co) with tosyl (p-toluenesulfonate) chloride in dry pyridine. To 28.7 g (1.5×10^{-1} mol) of tosyl chloride, 15.3 g (8.35×10^{-2} mol) of P1 dissolved in 100 mL of dry pyridine was added and left at room temperature for 48 hours. After this period, 10 g of KOH dissolved in 250 mL of water were added, and the mixture was heated up to 80°C. After cooling, the product was extracted with sulfuric ether, washed with diluted HCl, diluted $NaHCO_3$ solution, water and dried over anhydrous Na_2SO_4. After evaporation of the solvent under reduced pressure 12.76 g (yield= 92%) of an orange powder was obtained and further purified in an alumina column using benzene as an eluent. The product (P2) showed a single spot in thin layer chromatography with following characteristics: M.P.= 62-63°C, M+ = 165; Elementary analysis found: C, 65.36% , H, 7.50% e N, 17.10 % ; calculated : C, 65.43% , H, 7.93% e N, 16.96%).

Step 2 : Synthesis of 2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-amine-4-cyano (P3).

In a 3 L round bottom flask, 600 mL of liquid ammonia, 9 g (5.44×10^{-2} mol) of P2 and 120 mL of water were added. The flask was tightly closed to maintain the mixture under pressure and left at room temperature. After 3 days, the ammonia was eliminated and the product extracted with chloroform. The crude product crystallized from ether-petroleum ether yielded 9.37 g (yield = 94%) of a yellow powder with the following characteristics: M+= 182; P.F. 84-85 °C; Elementary analysis found: C 58.98%; H, 8.37%; N, 22.21%; calculated ($C_9H_{16}ON_3$): C, 59. 31%; H, 8.85 % ; N, 23.06 %).

Step 3 - Synthesis of 2,2,5,5-tetramethylpyrrolidine-1-oxyl-3-amino-4-carboxylic-acid (P4) (Poac).

8 g (4.38×10^{-2} mol) of P3 and 40 g of $Ba(OH)_2$ were suspended in 600 mL of water and added to a 3 L round bottom flask. The flask was tightly closed and heated up to 120°C for 90 minutes. After cooling, the mixture was neutralized with an excess of dry ice

and filtered. The aqueous solution was concentrated under reduced pressure and yielded 8

g of crude product (yield: 90%) that was crystallized from 90% ethanol. The product

presented the following characteristics: M.P. = 212°C (melts with sublimation); M+ = 201

(Figure 2), single peak in HPLC (Figure 3); elementary analysis found : C, 53.1% ; H, 8.28

5   %; N, 13.95 % ; calculated ($C_9H_{17}N_2O_3$): C, 53.71 % ; H, 8.52; N, 13.92 Infra-red (KBr) :

cm^{-1} : 3084, 2872, 2792, 2548 and 2132 (NH+3); 1643 v_{AS} (NH+3) ; 1574 (v_{AS} , C=O);

1456 (δ CH3); 1396 and 1376 (gem-dimethyl and COO-); 782 (δ C=O). Figure 4A shows

the EPR spectrum of POAC in aqueous solution, pH 5. The calculated values for the two

rotational correlation times (τ_B and τ_c) were 0.509 x 10^{-10} and 0.597 x 10^{-10}, respectively.

10   **Preparation of Fmoc-Poac**

201 mg (1 mmol) of P4 was dissolved in 1.5 mL of water in presence of 286 mg of

sodium carbonate.10H$_2$O in which 337 mg (1 mmol) of Fmoc-succinimidyl-carbonate

dissolved in 1.5 mL of acetone was added drop-wise. The reaction was carried out at room

temperature with stirring, and the pH was maintained around 9 by addition of sodium

15   carbonate. After 3 hours, the mixture was diluted with 25 mL of water and acidified with 1

N HCl until it reached pH 2. The desired product was extracted with ethyl acetate, washed

with small portions of water, dried over anhydrous sodium sulfate. After filtration, the

solvent was evaporated under reduced pressure. The crude product was crystallized twice

with chloroform and yielded 380 mg (yield : 90%). The product was characterized by mass

20   and infra-red spectroscopy, elementary analysis and EPR. Characteristics: M+ = 423

(Figure 5); elementary analysis found: C, 67.9 % ; H, 6.35 %; N, 6.60 ; calculated

($C_{24}H_{27}O_5N_2$): C, 68. 08% ; H, 6.28 % ; N, 6.62%; IR (KBr) cm^{-1}: 3444-3338 (broad band

OH and -CONHR); ~3030 (vAr CH); 3000-2700 (vAr COOH); 1723 (R-O-C-ON- and

COOH); 1543 (δ_{NH} and vCN); 1450 (δ CH3); 1235-1150 (gem-dimethyl group). The EPR

25   spectra of Fmoc-Poac in dimethylformamide is represented in Figure 4B and the calculated

τ_B and τ_c values are 1.14 x 10^{-10} s.rad^{-1} and 1.79 x 10^{-10} s. rad^{-1}, respectively.

Synthesis of Poac[7]-angiotensin II

Angiotensin II analogue labeled with the spin probe Poac (Asp-Arg-Val-Tyr-Ile-His-Poac-Phe) was synthesized in 0.15 mmol scale, by the solid phase method already mentioned and with alteration to provide the insertion of this marker in the middle of the

5   peptide chain. [J. Am. Chem. Soc. *115*, 11042 (1993)]. Fmoc-Phe-Wang-resin [J. Am. Chem. Soc. *95*, 1328 (1973)] with 0.41 mmol/g substitution degree acquired commercially was used. All couplings were carried out using 2.5 fold excess for the Fmoc-amino acids and 3 fold excess for Fmoc-Poac. The acylating reagents for coupling were diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole in a

10   dichloromethane:dimethylformamide mixture (1:1, v/v) as a solvent. The Fmoc deprotection was performed with 20% piperidine in dimethylformamide (v/v) for 20 min.

Interestingly, this synthesis demonstrated that the pyrrolidine structure of the Poac spin label allowed much easier incorporation of the subsequent amino acid residue than has been observed during the Toac[7]-AII synthesis. In this latter case, repetitive recoupling

15   reactions were necessary, including an increase in temperature. These procedures were not necessary in the case of the Poac derivatives, thus demonstrating that the Poac amine group reactivity is much higher than that of Toac.

After the completion of the synthesis, the peptide was cleaved from the resin in anhydrous HF containing 10% of a p-cresol and dimethylsulfide mixture, at 0°C for 90 min.

20   The crude peptide obtained after extraction and lyophilization (125 mg) was dissolved in 70 mL of water and the pH was raised to 10 with ammonium hydroxide and stirred for 2 h, to reverse the nitroxide protonation that occurs during the HF treatment. After lyophilization, the peptide was purified by preparative HPLC (high performance liquid chromatography) using a reverse phase C_{18} (25 x 250 mm) and ammonium acetate 0.02 M,

25   pH 5 and acetonitrile 60% in water, as solvents A and B, respectively. The linear gradient applied was from 20-65% of B for 135 min.

The homogeneity of the peptide was confirmed through analytical HPLC (Figure 6), mass spectrometry, M+ = 1132.55 (Figure 7), and the amino acids analysis showed the expected composition : Asp 0.95 (1.00); Val 0.96 (1.00); Ile 1.20 (1.00); Tyr 1.02 (1.00); Phe 1.00 (1.00); His 0.96 (1.00); Arg 1.02 (1.00). Figure 8 displays the EPR spectra of

5   0.25 mM Poac[7]-AII at pH 3, 6 and 9 aqueous solution. No significant variation on the rotational correlation time values was observed for this paramagnetic AII analogue thus suggesting that its conformation is not affected by the pH of the media.